Inositol-phospholipid formation in purified synaptosomes and mitochondria

A

Characterisation of the Molecular Basis for Dopamine Receptor Subtype Diversity.

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The D2 dopamine receptor belongs to a diverse family of receptors which interact with guanine nucleotide-binding proteins (G-proteins) in coupling to various intracellular second messenger systems. Each of the G protein coupled receptors which have been identified to date share a high level of structural homology, with seven putative transmembrane domains. We have shown by molecular cloning and polymerase chain reaction analysis (PCR) that at least two structural forms of the D2 receptor exist. These D2 receptor subtypes arise from alternate splicing of a single D2 receptor gene and differ by the insertion of 29 amino acids in the putative third cytoplasmic loop. In other members of the G protein-coupled receptor family (including the muscarinic and adrenergic receptors) this region of the molecule has been implicated in coupling the receptor to specific intracellular signalling pathways. We are therefore examining whether these structural subtypes represent functionally distinct forms of the receptor. To this end the "long", D2A, and "short", D2B, receptor subtypes have been stably expressed in a Chinese Hamster Ovary cell line (CHO K1). The D2A and D2B receptor subtypes have a similar pharmacological profile for the D2 agonists dopamine and PPHT and the antagonists spiperone and haloperidol. Dopamine binding to either receptor subtype inhibits endogenous Calcitonin Gene Related Peptide (CGRP) receptor stimulated cAMP production. This result, and the fact that a guanyl nucleotide reduces the affinity of the D2 receptors for dopamine, is consistant with both of the D2 receptors coupling to a member of the Gi family of G-proteins in this cell line. Dopamine stimulation of the two subtypes also gives rise to an immediate increase in intracellular calcium due to both calcium mobilisation from intracellular stores and influx from the extracellular medium. To determine if this calcium response is due to activation of phosphoinositide (PI) turnover, we are also assessing the ability of both of the D2 receptors to stimulate inositol phosphate generation. These comparisons should allow us to determine whether the structural diversity of the D2A and D2B dopamine receptor subtypes has any functional significance.

B

MUSCARINIC RECEPTOR-STIMULATED PHOSPHOINOSITIDASE C ACTIVITY IN CEREBRAL CORTICAL MEMBRANES

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Using a particulate fraction from mouse or guinea pig cerebral cortical slices pre-labelled with [^{3}H]inositol, we have been able to demonstrate phosphoinositidase C (PIC) activity which is stimulated by a number of agents in a similar fashion in either species. Increasing the free calcium ion concentration to a pCa < 7 led to a concentration-dependent increase in PIC activity, such that at 1 mM free calcium, 12.8 ± 1.0 % of radiolabelled phosphoinositides were converted to [^{3}H]inositol phosphates. At pCa 6.5, guanosine 3-thiotriphosphate (GTP-γ-S) produced a concentration-dependent increase in PIC activity with an EC_{50} value of 0.6 ± 0.1 μM and a maximal response of 3.1 ± 1.4 %. GTP-γ-S-, but not Ca^{2+}-elicited responses were dependent on the presence of deoxycholate (1 mM) in the incubation medium. The muscarinic agonist carbachol also stimulated PIC activity in a GTP-γ-S-dependent (10 μM), atropine-sensitive manner with an EC_{50} value of 46 ± 17 μM and a maximal response of 0.8 ± 0.3 %. These studies support the proposed intermediacy of a GTP-binding regulatory protein in the stimulation of PIC activity by muscarinic receptor activation.

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C

ACTIVATION OF PHOSPHOLIPASE C IN BLEACHED ROD OUTER SEGMENT MEMBRANES: A POSSIBLE ROLE FOR 48 K PROTEIN

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Bovine rod outer segments (ROS) prepared on a continuous sucrose gradient were bleached with or without ATP (3 mM) and GTP (1 mM) for 10-20 mins at 37°. ROS membranes were then spun at 100,000 g for 15 min, and phospholipase C (PLC) activity in the membraneous pellet was assayed using exogenously added [^{3}H]-phosphatidylinositol 4,5-bisphosphate (PIP_2) vesicles as substrate. PLC activity in ROS membranes bleached in the presence of nucleotides was 2-7 fold higher than membranes bleached in the absence of nucleotides. This difference in PLC enzyme activity was observed at very dilute protein concentrations (1-10 μg of ROS protein). Moreover, the increased PLC activity in ROS membranes was evident at 1.6 μM, 10 μM, and 600 μM free Ca^{+2}. Two years ago, we reported that a high salt extract of bleached ROS membranes contained significant PLC activity (1). The major protein (>80%) in these extracts was the 48 K protein. Immunoblots of such extracts with a peptide specific anti-PLC that recognizes two isozymes of PLC in ROS (2) did not detect any PLC antigen in these preparations. These results suggested that the high salt extracts of bleached ROS contained residual PLC enzyme protein and that the observed enrichment of PLC activity in these extracts was due to the presence of an activator protein in these preparations. Because the major protein in these preparations was the 48 K protein, we decided to test the effect of 48 K (3) on PLC activity in ROS membranes. Addition of 48 K (0.25-1.0 μg) to bleached ROS membranes in the presence of ATP and GTP activated ROS PLC 80-370%. Boiled 48 K failed to activate ROS PLC. Our data indicate: 1) ROS membranes contain a very active PLC that is regulated by both ATP and GTP and 2) this regulation of PLC by nucleotide triphosphates may involve the 48 K protein, as demonstrated by its ability to increase PLC activity in bleached ROS membranes.

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D

INOSITOLPHOSPHOLIPID FORMATION IN PURIFIED SYNAPTOSOMES AND MITOCHONDRIA: EVIDENCE FOR A NOVEL POLYPHOSPHOINOSITIDE.

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The present study investigates the subcellular distribution of endogenous (poly)-phosphoinositide kinases in rat brain tissue subjected to differential centrifugation. Kinase activities were studied by incubation (10 sec) of lysed fractions under hypotonic conditions in phosphorylation buffer containing 7.5 μM ^{32}P-ATP. This incubation system has been shown to favour the formation of phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5 bisphosphate (PIP_2), and phosphatidic acid (PA); (Bothmer et al., Neurochem. Int. 1990:17(1), 27-32). Subcellular differentiation was performed using a discontinuous Percoll gradient on an S1 fraction. Five fractions were formed; fraction 1 was enriched in unidentified membrane material; fraction 2 in myelin, fraction 3 and 4 in progressively larger synaptosomes with intrasynaptosomal mitochondria. Fraction 5 was enriched in extra synaptosomal mitochondria. The formation of PIP was enriched progressively with the state of purification of the synaptosomes (2.5 pmol/mg protein in fraction 1 and 2; 3.2 pmol/mg protein in fraction 3; 5.5 pmol/mg protein in fraction 4; 4.1 pmol/mg protein in fraction 5). PIP_2 formation showed similar enrichment in synaptosomes (0.8 pmol/mg protein in fraction 1 to 1.4 pmol/mg protein in fraction 4; 0.8 pmol PIP_2/mg protein in fraction 5). Unexpectedly, a fourth radiolabelled phospholipid was found in fraction 5, and to a lesser extent also in fraction 4. The amounts of this novel substance were similar to those found for PIP_2. Control experiments with purified mitochondria (prepared using a discontinuous flotation Ficoll/sucrose gradient) upon phosphorylation showed the same novel phospholipid. This phospholipid had a RF value on TLC somewhat smaller than that of PIP_2 and similar to PIP3 as found in neutrophils (Traynor-Kaplan, Nature: 334 (1988), pp 353-356). If the novel phospholipid in brain indeed turns out to be PIP_3, this would suggest that PIP_2 kinase activity is present in mitochondria. Follow-up research is presently undertaken into identifying this novel phospholipid. In conclusion, the present data favour use of the assay system with gamma-^{32}P-ATP and short incubation periods in the study of the rapid intra-conversion of the inositolphospholipids in vitro.